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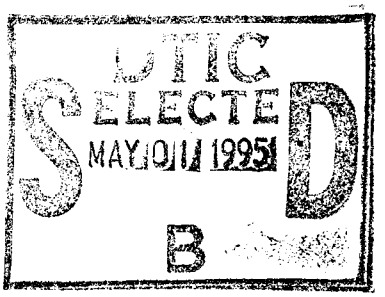
CONTROL OF *BACILLUS STEAROTHERMOPHILUS* AND *BACILLUS COAGULANS* IN LOW-ACID FOOD WITH FOOD-GRADE ADDITIVES

by

Anthony Sikes, Ph.D.



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13. ABSTRACT *(Maximum 200 words)*

Results from previous antimicrobial studies with sucrose laurate (50 ppm) and the combination preservative system of SLEB [sucrose laurate (SL) + tert-butylated hydroxyanisole], 60 ppm, indicted that *Bacillus strearthermophilus* spore germination/outgrowth was inhibited, during 144 h of storage at 55°C on AAMS (antibiotic assay agar + soluble starch, pH 6.8) Laboratory media containing SL or SLEB. The present investigation with SL and SLEB showed that similar levels of spore inhibition (outgrowth) of *B. Stearothermophilus* and *B. coagulans* occurred when SLEB was added to a mixed vegetables/water puree (1:1, w/w) at pH 5.2-5.3. Previous results had indicated that the minimum inhibitory concentration (MIC) of SL required to prevent germination/outgrowth of *B. coagulans* was more than 700 ppm, while *B. stearothermophilus* exhibited a greater sensitivity towards SL, e.g., MIC: >60 ppm in Laboratory media. At concentrations of 0, 100, 200, 400 and 800 ppm, LS had no apparent effect on the germination/outgrowth activities of *B. coagulans* or *B. stearothermophilus*, during 96 and 72 h of storage at 50° and 55°C, respectively. However, SLEB, at 100 ppm, prevented spore outgrowth of both *B. coagulans* and *B. stearothermophilus*, during a similar storage time and temperature. Thus, it was concluded from this study that it might be feasible to use SLEB in certain military rations (low-acid food items) to control thermophilic spoilage and reduce thermal processing requirements without compromising food safety.

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TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

PREFACE

This investigation discusses the results of a study designed to determine the efficacy of a new chemical preservative system, which is intended to be used to control thermophilic spore activity in low-acid canned ration items. This system consists of food-grade food additives, such as, sucrose laurate (SL), ethylenediaminetetraacetate (E) and butylated hydroxyanisole (B) used in combination (1:1:1, v/v/v). Specifically, this investigation evaluated the effectiveness of SL and SLEB, when added to mixed vegetables/water puree (1:1) containing typical thermophilic, spoilage bacteria (Bacillus stearothermophilus or B. coagulans), to control or inhibit microbial activity.

This work was performed under the work unit titled "Microbiological Validation," Project # AH99BBCOO, during the period of October 1993 through September 1994.

Control of *Bacillus stearothermophilus* and *Bacillus coagulans* in Low-Acid Food with Food-Grade Additives

INTRODUCTION

Sucrose fatty acid esters are used in the food industry as emulsifying agents in various food systems. In addition to their primary function in food, they have been shown to control thermophilic spore activity in canned foods (Kabara, 1982; Tsuchido et al., 1987; Nakayama et al., 1982; Suwa et al., 1986). Although the mechanism of sucrose ester inhibition is not well understood, evidence presented in the present investigation shows that bacterial spores are particularly sensitive to the presence of sucrose esters, especially if food additives, such as ethylenediamine-tetraacetate (EDTA) and/or butylated hydroxyanisole (BHA), are present.

The concept of using combinations of multifunctional food-grade inhibitors to control microbial activity in food ("systems approach") was first proposed by Kabara (1981). The basic idea of the systems approach to food preservation is the creation of a microbially hostile environment, which precludes the proliferation of undersirable microorganisms (Kabara, 1979).

The advantage of this approach is that presently approved multifunctional food additives, which are either approved for food use or have GRAS (Generally Recognized As Safe) status, e.g., parabens, sorbic acid, etc., can be used to design preservation systems that will meet specific microbiological requirements.

In the Kabara's (1979) system, three food-grade additives were used in combination: monolaurin (fatty acid), BHA/BHT (butylated hydroxytoluene; food-grade phenolic antioxidant) and EDTA (chelator). Each component of the preservative system has a specific effect on the microbial cell. In the present investigation, a modified version of Kabara's system was evaluated. Multifunctional sucrose laurate (SL) was substituted for monolaurin, while the other components of the system, EDTA and BHA, remained unchanged.

Thus, the objective of this investigation was to evaluate the ability of sucrose laurate alone and in combination with other antimicrobial agents to prevent germination/outgrowth of B. stearothermophilus and B. coagulans in a mixed vegetables puree.

MATERIALS AND METHODS

Spore preparation. Stock cultures of Bacillus coagulans (ATCC 8038) and Bacillus stearothermophilus (ATCC 12980) spores were obtained from the culture collection of the Microbiology Section, U. S. Army Natick RD&E Center, Natick, MA. Spores of B. stearothermophilus and B. coagulans were prepared according to the procedures described by Feeherry et al. (1987) and Yokoya and York (1965), respectively. After washing and cleaning (0.05M phosphate buffer, pH 7.2), the spore densities of B. stearothermophilus and B. coagulans were determined on antibiotic assay medium supplemented with 0.1% soluble starch (AAMS, Difco; Feeherry et al., 1987) and thermoacidurans agar supplemented with 0.1% soluble starch (Difco; Lynch and Potter, 1988), respectively. Subsequently, washed and cleaned spores were resuspended in sterile phosphate buffer (pH 7.2) and stored at 4°-5°C until used. The final spore density was ~106-108 spores/mL.

Additives. Stock solutions of sucrose laurate (L- 1695; Mitsubishi-Kasei America Inc., White Plains, NY) and BHA (2[3]-t-butyl-4-hydroxyanisole; Sigma Chemical Company, St. Louis, MO) were prepared by suspending 1 g of additive in 10 mL of absolute ethyl alcohol (Quantum Chemical Corp., Tuscola, IL).

Solutions of disodium ethylenediaminetetraacetate [EDTA], Fisher Scientific Co, Fair Lawn, NJ) were similarly prepared, except deionized water was used as the suspending medium. After preparation, all stock solutions were stored (1°-4°C) in screw-capped tubes tightly sealed and wrapped with parafilm (American National Can, Greenwich, CT) until they were used. Fresh solutions were prepared for each experimental run.

Minimum inhibitory concentrations (MIC). The procedure that was used to determine the minimum inhibitory concentration of sucrose laurate (SL), EDTA (E), BHA (B) and SLEB required to prevent germination/outgrowth of B. coagulans and B. stearothermophilus spores was similar to the procedure described by Bargiota et al. (1987). Prior to performing all experiments, spores were heat activated in phosphate buffer (pH 7.2) by heating at 100°C for 10 min (B. stearothermophilus) or 5 min (B. coagulans) in a cabinet with free-flowing steam (G. H. Wahmann Manufacturing CO., Baltimore, MD), Feeherry et al. (1987).

Heat-activated spores of the test organisms were serially diluted in phosphate buffer (pH 7.2) to yield ~10^3 and 10^5 spores/mL. One-tenth mL aliquots of these spores suspensions were spread plated with a bent glass rod on thermoacidurans (B. coagulans) and AAMS agars (B. stearothermophilus) in triplicate.

Agar plates containing each antimicrobial agent, SLEB (1:1:1, v/v/v), SL, EDTA and BHA, were prepared by adding an appropriate volume of each stock solution to 100 mL of melted nonsterile agar, thermoacidurans and AAMS, and sterilizing at 121°C for 15 min at 15 psi. The final pH of the sterile agar was pH 6.5-6.8. The concentration of each antagonist ranged from 25 to 700 ppm.

Preparation of mixed vegetables puree. Tray packs (TP) of mixed vegetables (Mealmakers, Inc., Roswell, NM) were prepared by aseptically opening and placing ~500 g portions of vegetables into a clean blender jar (Waring Products Div., Dynamics Corp. of America, New Hartford, CT) and adding an equal portion of deionized water (1:1, w/w). The mixture was blended at high speed for 5 min and subsequently dispensed in 100 mL quantities into 500 mL Erlenmeyer flasks. To this mixture, different levels of the stock inhibitors were added (0 to 700 ppm) before sterilization at 121°C for 15 min at 15 psi.

Growth experiments. To 100 mL of the antimicrobial-containing vegetable puree, 1 mL aliquots of the heat-activated spore suspension was added (~10^3 to 10^4 spores/mL). Samples were incubated, unshaken, in a model 3916 Forma Scientific Incubator (Marietta, OH) up to 96 h at 55°C, B. stearothermophilus, or 50°C, B. coagulans.

Growth activities were monitored by removing appropriate aliquots at 0, 8, 12 or 24 h intervals, serially diluting (pH 7.2 phosphate buffer) and spread plating on thermoacidurans or AAMS agar. Inoculated plates were incubated at 55°C for 24 h, B. stearothermophilus, or 50°C 48-72 h, B. coagulans.

pH determination. At each sampling interval, two samples of the pureed vegetables mix was taken from each replicate for pH measurement. Measurements were obtained with a Orion Expandable ionAnalyzer, Model EA 920 pH meter equipped with a Ross 8135 pH/Reference surface electrode (Orion Research, 380 Putnam Ave., Cambridge, MA). Before measurements were taken, the temperature of the mix was allowed to equilibrate to ambient temperature (~21° to 25°C).

Statistical analysis. Difference in microbial counts and magnitude of pH changes were examined for statistical significance, using the one-way analysis of variance procedures of Statgraphics^{tm} (Statistical Graphics Corp., Rockville, MD). Bacterial plate count data represent mean $\log_{10}$ colony-forming units/mL of two replications with three samples/replication ($n = 6$). Significant differences ($p < 0.05$) between treatment means were separated by Least Significant Difference procedures (Dowdy and Wearden, 1983).

RESULTS

The minimum inhibitory concentrations of sucrose laurate (SL), EDTA (E), BHA (B) and SLEB required to inhibit germination/outgrowth of B. stearothermophilus and B. coagulans are shown in Figures 1 and 2, respectively.

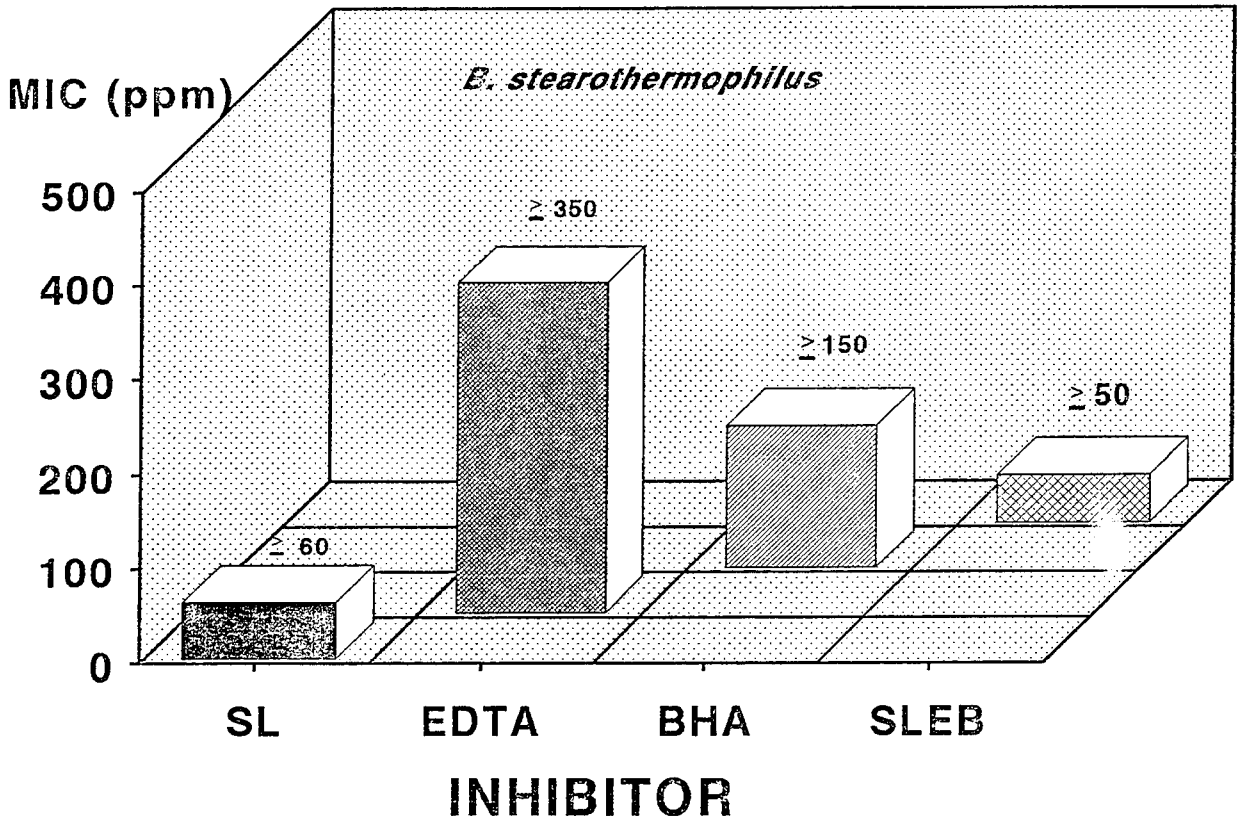

Figure 1. Minimum inhibitory concentrations (MIC) of sucrose laurate (SL), EDTA (E), BHA (B) and SLEB (1:1:1, v/v/v) required to prevent germination and outgrowth of spores of *Bacillus stearothermophilus.* Numbers above bars indicate specific MIC values for that inhibitor.

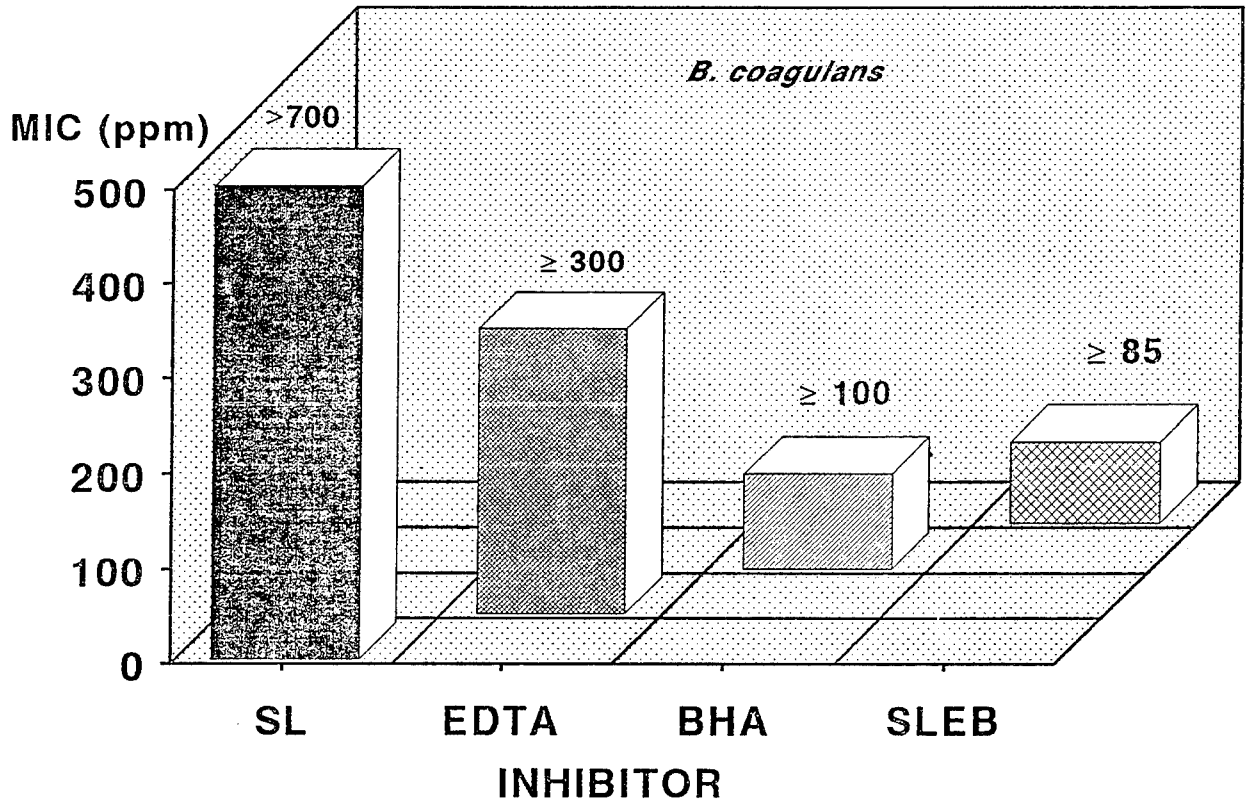

Figure 2. Minimum inhibitory concentrations (MIC) of sucrose laurate, EDTA, BHA and SLEB required to prevent the germination and outgrowth of *Bacillus coagulans* spores. Numbers above bars indicate the specific MIC value for that inhibitor.

The results indicated that on laboratory media germination of B. stearothermophilus and B. coagulans spores was more sensitive to SLEB (>50 and >85 ppm, respectively) than any of its components: SL = >60 and >700 ppm; EDTA = > 350 and > 300 ppm; BHA = > 150 and > 100 ppm, respectively.

The ability of B. stearothermophilus and B. coagulans to germinate and grow in a mixed vegetables puree (pH 5.3 - 5.4) containing several concentrations of SL and SLEB (100, 200, 400 and 800 ppm) was evaluated during storage at 55°C and 50°C, respectively. SL, up to 800 ppm, had no apparent effect on the growth activites of B. stearothermophilus or B. coagulans during storage for 72 and 96 h, respectively (Figures 3 and 4).

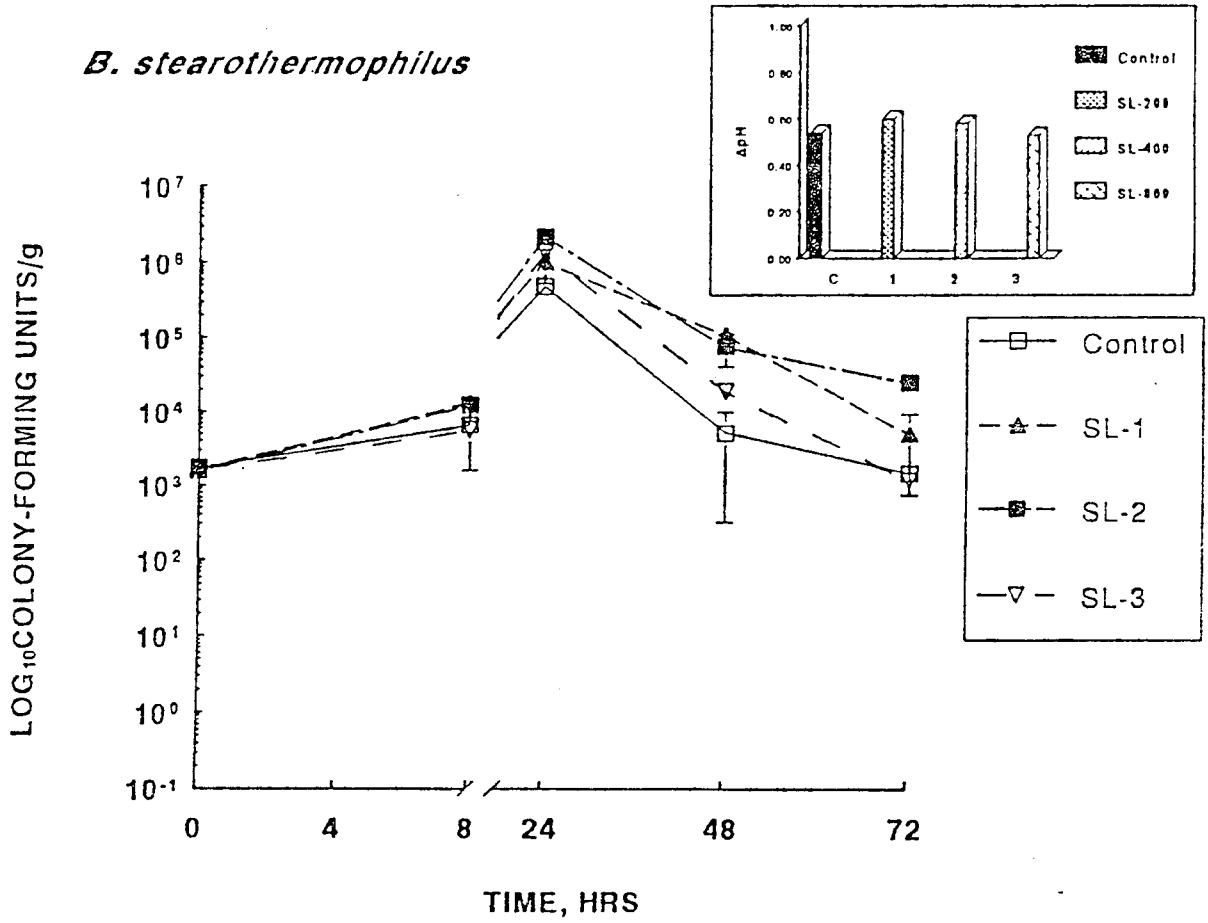

Figure 3. Effects of sucrose laurate (SL) on the germination and outgrowth of *B. stearothermophilus* spores cultured in a mixed vegetables/water puree (1:1, w/w; pH 5.35) at 55°C. Each point represents the mean of six observations (3 samples/rep) ± SD. Inset indicates the average pH change (Δ pH) during the course of the experiment (n = 6). SL concentrations used were 200 (SL-1), 400 (SL-2) and 800 ppm (SL-3).

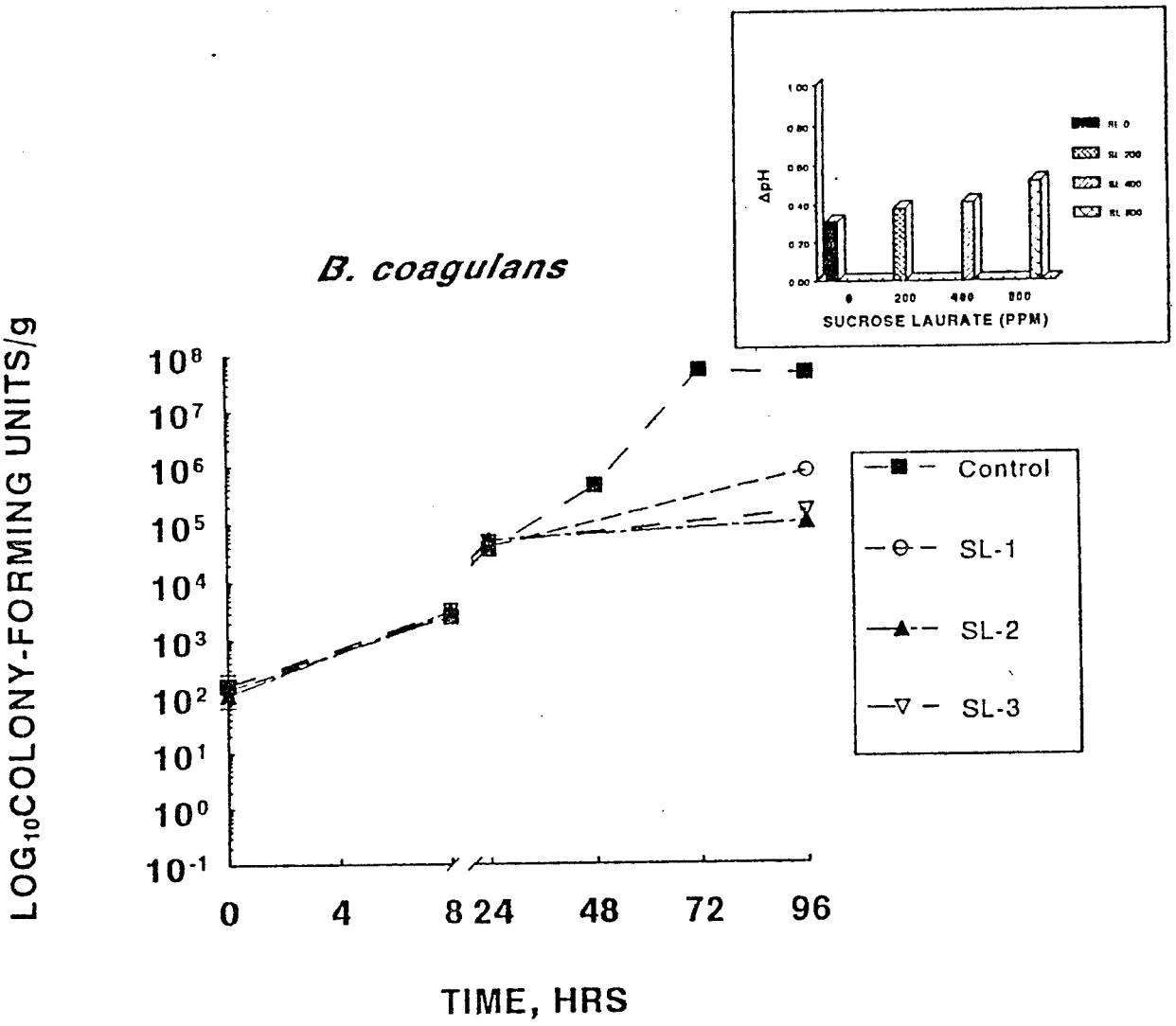

Figure 4. Effects of sucrose laurate (SL) on the germination and outgrowth of *B. coagulans* spores cultured in a mixed vegetables/water puree (1:1, w/w; pH 5.35) at 55°C. Each point represents the mean of six observations (3 sample/rep) ± SD. Inset indicates the average pH change (Δ pH) during the course of the experiment (n = 6). SL concentrations used were 200 (SL-1), 400 (SL-2) and 800 ppm (SL-3).

However, SLEB, at 100 ppm (Figures 5 & 6), inhibited the growth activities of both sporeformers under the same growth conditions as described above, Figures 3 and 4. It is also apparent from Figure 5 that between 24 h and 96 h the survival of B. stearothermophilus in the control sample decreased at a rate faster than any of the treated samples. It is believed that this observation is due to the phenomenon known as "autosterilization" or "spore deactivation", a characteristic often associated with thermophilic flat sour spoilage bacteria. Autosterilization is assumed to occur when a spoilage condition exists and no viable organisms can be recovered by cultural methods. Thus, it is assumed that the spoilage organism self-destructs because of the build-up of metabolic end products (Pearce and Wheaton, 1952; Schmidt and Nank, 1957).

Although not conclusively demonstrated in the present investigation, it does appear that all components of SLEB are necessary for it to function effectively in a food system; however, additional research is necessary in order to establish this fact.

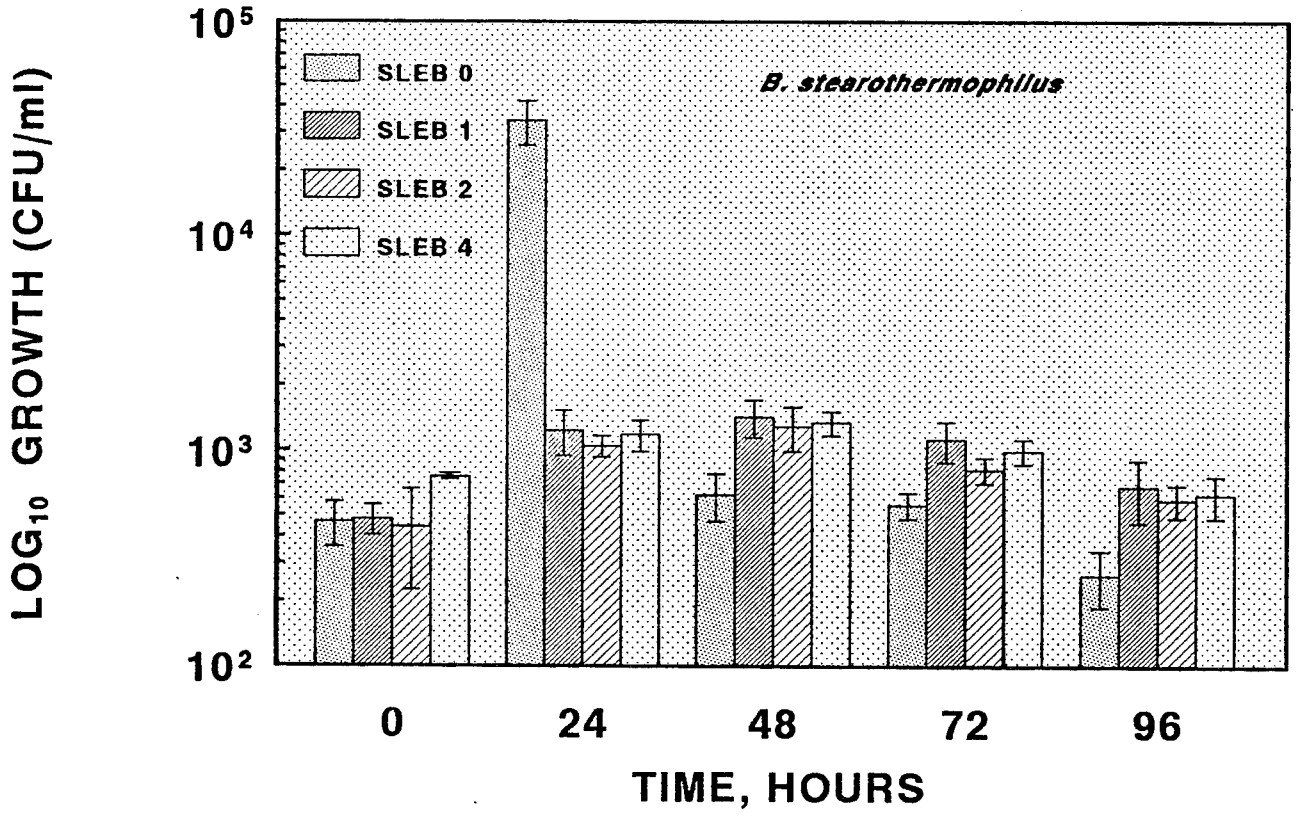

Figure 5. Inhibitory effect of SLEB (sucrose laurate + EDTA + BHA; 1:1:1, v/v/v) on the germination and outgrowth of *B. stearothermophilus* spores when cultured in a mixed vegetables puree (pH 5.35) at 55°C. SLEB concentrations were 0 = control, 1 = 100 ppm, 2 = 200 ppm and 4 = 400 ppm. Bar heights represent the mean of two experimental runs with three samples/run. Error bars denote the standard deviation, n = 6.

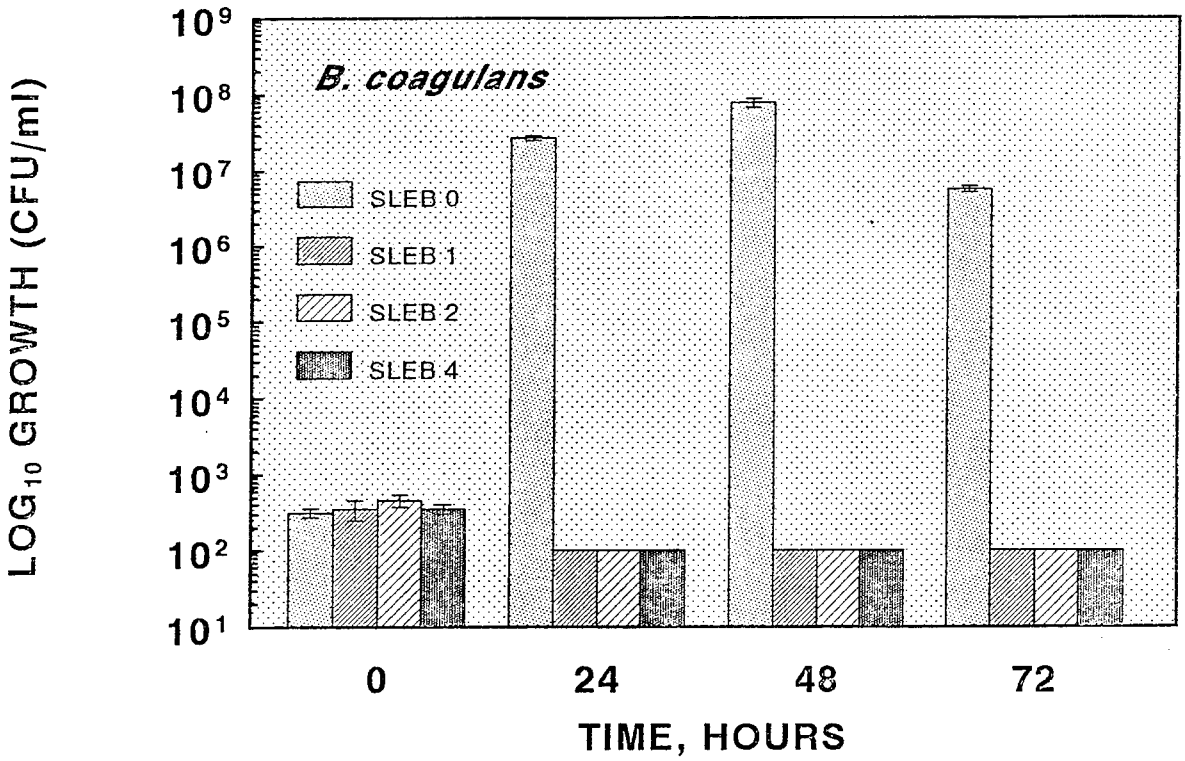

Figure 6. Inhibitory effect of SLEB (1:1:1, v/v/v) on the germination and outgrowth of *B. coagulans* when cultured in a mixed vegetables puree (pH 5.35) at 50°C. SLEB concentrations were 0 = control, 1 = 100 ppm, 2 = 200 ppm, and 4 = 400 ppm. Bar heights represent the mean of two experimental runs with three samples/run. Error bars denote the standard deviation, n = 6.

Mean pH differences were plotted against SLEB concentrations (Figures 7 and 8). The control pH values for both B. stearothermophilus and B. coagulans differed significantly ($p < 0.05$) from the treated mixed vegetables puree samples; however, differences between treated samples were not significant ($p > 0.05$).

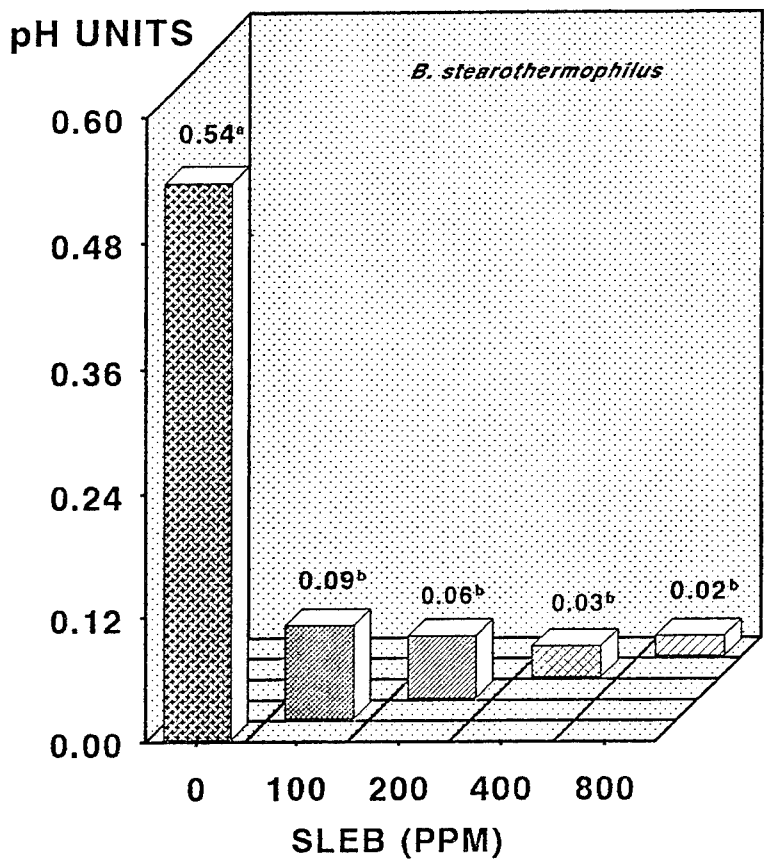

Figure 7. Mean pH change during the growth of *B. stearothermophilus* in mixed vegetables puree containing different concentrations of SLEB (pH 4.88 - 5.38). Mean values with common superscripts are not significantly different ($p > 0.05$; $n = 4$).

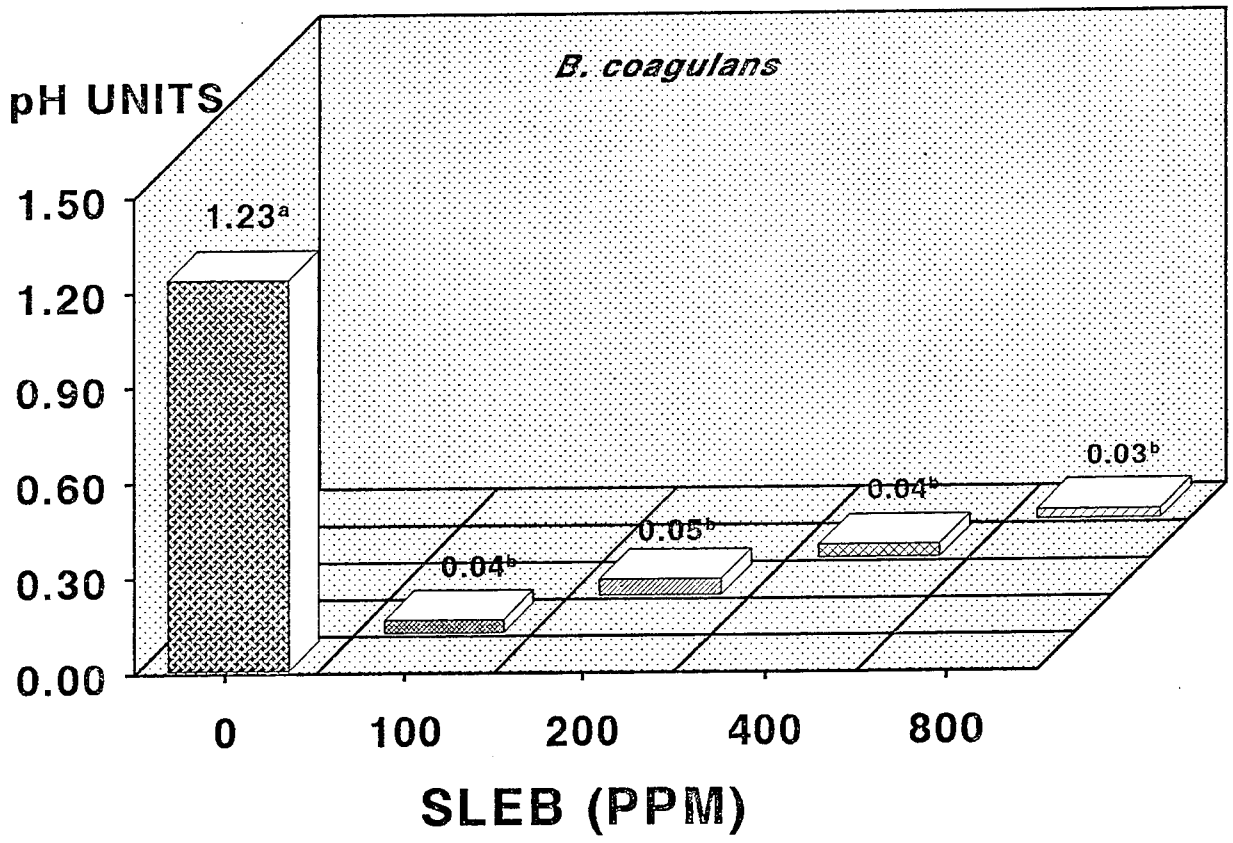

Figure 8. Mean pH change during the growth of *B. coagulans* in a mixed vegetables puree containing different concentrations of SLEB (pH 4.33 - 5.33). Mean values with common superscripts are not significantly different ($p > 0.05$; $n = 4$).

CONCLUSION

It was demonstrated in this investigation that, in a complex food system, such as pureed mixed vegetables, SLEB was an effective antimicrobial agent against the germination/outgrowth of B. stearothermophilus and B. coagulans spores. In laboratory media, it was also demonstrated that SL, at a MIC of 60 ppm, inhibited germination/outgrowth of B. stearothermophilus spores but had no apparent effect on the germination/outgrowth B. coagulans spores (MIC >700 ppm). In a pureed mixed vegetables food system, SL, at 800 ppm, exerted no inhibitory effect on B. coagulans or B. stearothermophilus.

REFERENCES

1. Bargiota, E., Rico-Munoz, E. and Davidson, P. M. 1987. Lethal effects of methyl and propyl parabens as related to Staphylococcus aureus lipid composition. Intl. J. Food Microbiol. 4:257-266.

2. Dowdy, S. and Wearden, S. 1983. Statistics for Research. John Wiley & Son, Inc. , New York, NY.

3. Feeherry, F. E., Munsey, D. T. and Rowley, D. B. 1987. Thermal inactivation and injury of Bacillus stearothermophilus spores. Appl. Environ. Microbiol. 53: 365-370.

4. Kabara, J. J. 1979. Multi-functional food-grade preservatives in cosmetics. Drugs and Cosmetics Ind., Oct., pp., 60-145.

5. Kabara, J. J. 1981. Food-grade chemicals for use in designing food preservation systems. J. Food Prot. 44:633-647.

6. Kabara, J. J. 1982. A new preservation system for food. J. Food Safety. 4:13-25.

7. Lynch, D. J. and N. N. Potter. 1988. Effects of organic acids on thermal inactivation of Bacillus stearothermophilus and Bacillus coagulans spores in frankfurter emulsion slurry. J. Food Prot. 51:475-480.

8. Nakayama, A., J. Sonobe and R. Shinya. 1982. Effects of sucrose esters of fatty acids on flat sour spoilage by obligate anaerobes. J. Food Hyg. Soc. Japan. 23:25-32.

9. Pearce, W. E. and E. Wheaton. 1952. Autosterilization of thermophilic spores in canned foods. Food Res. 17:487-494.

10. Suwa, N., H. Kubota, H. Takahashi and H. Machida. 1986. Effects of sucrose fatty acid esters on the spoilage of canned coffee caused by thermophilic spore-forming bacteria. J. Jpn. Soc. Food Sci. Technol. 33:44-51.

11. Schmidt, C. F. and W. E. Nank. 1957. Sterilization by means of spore deactivation. Food Res. 22:562-566.

12. Tsuchido, T., Y. Ahn and M. Takano. 1987. Lysis of Bacillus subtilis cells by gycerol and sucrose esters of fatty acids. Appl. Environ. Microbiol. 53:505-508.

13. Yokoya, F. and G. K. York. 1965. Effect of several environmental conditions on the "thermal death rate" of endospores of aerobic, thermophilic bacteria. Appl. Microbiol. 13:993-999.

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